Lung Immune Tone Regulation by the Gut-Lung Immune Axis: Short-chain Fatty Acid Receptors FFAR2 and FFAR3, and IL-1 β Expression Profiling in Mouse and Human Lung

Qing Liu[∞], Xiaoli Tian, Daisuke Maruyama, Mehrdad Arjomandi^{**}, and Arun Prakash[¶]

Department of Anesthesia and Perioperative Care, University of California San Francisco and San Francisco General Hospital, San Francisco, CA; ^{**} Department of Medicine, University of California San Francisco and Medical Section, San Francisco VA Medical Center; [∞] Current address: Department of Anesthesiology, Peking Medical College, Beijing, China.

[¶] Corresponding Author:

Arun Prakash, M.D., Ph.D., Department of Anesthesia and Perioperative Care; San Francisco General Hospital, University of California, San Francisco; Phone: 415-502-7095; Fax: 415-476-5946; email: arun.prakash@ucsf.edu

Department/Institution to which the work is attributed: Department of Anesthesia and Perioperative Care, University of California San Francisco and San Francisco General Hospital, San Francisco, California.

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1 **ABSTRACT**

2 Microbial metabolites produced by the gut microbiome, such as short-chain fatty
3 acids (SCFA), can influence both local intestinal and distant lung physiology and
4 response to injury. However, how lung immune activity is regulated by SCFAs is
5 unknown. We examined fresh human lung tissue and observed the presence of
6 SCFAs with large inter-individual and even intra-lobe variability. *In vitro*, SCFAs
7 were capable of modifying the metabolic programming in both resting and LPS-
8 exposed alveolar macrophages (AM). Additionally, since we hypothesized that
9 lung immune tone could be defined through priming of the inflammasome (aka
10 signal 1), we interrogated naïve mouse lungs for pro-IL-1 β message and
11 localized its presence within the alveolar space *in situ*, specifically in AM subsets,
12 and in close proximity to alveolar type 2 epithelial (AT2) cells. We established
13 that metabolically active gut microbiota, that produce SCFAs, can transmit LPS
14 and SCFAs to the lung (potential sources of signal 1), and thereby could regulate
15 lung immune tone and metabolic programming. To understand how murine lung
16 cells sensed and upregulated IL-1 β in response to gut-microbiome factors, we
17 determined that *in vitro*, AM and AT2 cells expressed SCFA receptors, FFAR2,
18 FFAR3, and IL-1 β but with different expression patterns and LPS-inducibility.
19 Finally, we observed that IL-1 β , FFAR2 and FFAR3 were expressed both in
20 isolated human AM and AT2 cells *ex-vivo*, but in fresh human lung sections *in*
21 *situ*, only AM expressed IL-1 β at rest and after LPS challenge. Together, this

1 translational study using mouse and human lung tissue and cells supports an
2 important role for the gut microbiome and SCFAs in regulating lung immune tone.

3

4 **INTRODUCTION**

5 The human gut and lung microbiomes have been linked to host health and
6 immune development (14, 22, 29, 32, 45). Key microbial metabolites such as
7 short-chain fatty acids (SCFA), which originate via microbiome fermentation or
8 from the diet, have been shown to regulate local tissue inflammation, and act as
9 communication signals to extra-intestinal organs (28, 43, 46). SCFAs can
10 influence allergic and inflammatory lung diseases including asthma (16, 21, 50,
11 53). However, the lung microbiome's low biomass (10^3 - 10^5 /g) (55) and lack of
12 significant metabolic activity imply that they are an unlikely source of SCFA
13 production. Consequently, we and other investigators have proposed that SCFAs
14 modulate lung immune function via direct or indirect mechanisms (7, 11, 50), but
15 thus far no consensus has been established on how gut-originating SCFAs
16 regulate lung inflammatory responses.

17 The lung likely exists in a state of immune responsiveness that is influenced
18 by numerous factors, including environmental exposures, genetics, diet,
19 medications, prior disease/injury exposure, and microbiome. Many of these
20 factors are complexly intertwined and likely contribute to a dynamic '*lung immune*
21 *tone*' that informs the host lung response to the next injury or infectious exposure.
22 Microbiome-sourced bacterial signatures, such as LPS, and metabolites, such as

1 SCFAs, could contribute to creating this lung immune tone within the gut-lung
 2 immune axis. The primary metabolite receptors for SCFAs are two free fatty acid
 3 receptors (FFAR): FFAR2 (GPR43) and FFAR3 (GPR 41) (6, 34, 37, 40). FFAR2
 4 is engaged primarily by acetate and propionate, and FFAR3 by propionate and
 5 butyrate (25, 47). Expression of these FFARs has been described in the
 6 intestinal stromal and immune cells, mainly in the colon, but FFAR2 and FFAR3
 7 have also been detected extra-intestinally (5, 28, 34).

8 We previously demonstrated that sensing of LPS and SCFAs produced by
 9 the gut microbiome could strongly influence the course of lung injury and
 10 infections, thereby supporting the existence of the gut-lung axis of immune
 11 communication (41, 50) . Moreover, we reported that co-exposure of bacterial
 12 products, such as LPS, with SCFAs enhanced or repressed inflammatory
 13 responses depending on their concentrations or levels within the lung (50)
 14 (reflecting the presence of specific types of gram-negative and gram-positive
 15 fermenting bacteria in the microbiome). We further reported low but detectable
 16 resting IL-1 β expression in lung tissue and interpreted this as gut-microbiome
 17 mediated inflammasome priming (signal 1) (50). The inflammasome function was
 18 also required for appropriate host response to lung injury and infection (51).

19 In the present study we characterized the pulmonary component of the gut-
 20 lung immune axis and elucidated how SCFA and microbial product presence and
 21 sensing in the lung may contribute to steady-state lung immune tone. To do so,
 22 we assessed the expression and regulation of FFAR2 and FFAR3 using mouse

1 cell lines and freshly isolated and cultured human lung cells. We also examined
2 intact mouse or human lung tissue to identify the cellular source(s) of IL-1 β ,
3 FFAR2 and FFAR3 expression. By identifying lung cell types that receive the gut-
4 derived signals (LPS, SCFAs, etc.) and thereby establish lung baseline IL-1 β
5 expression, we hope to advance our understanding of the potential interactions
6 between the gastrointestinal and pulmonary systems in health and disease.

7

8 **MATERIALS AND METHODS**

9 **Animal care**

10 All studies were approved by the institutional animal care and use
11 committee at University of California, San Francisco (Protocol# AN152943). All
12 mice were purchased (The Jackson Laboratory, Bar Harbor, ME) or bred in the
13 animal facility at University of California, San Francisco. Wild-type (WT) C57BL/6,
14 C3H/HeO_uJ mice, and germ-free (GF aka gnotobiotic) mice were used in this
15 study (8-10 weeks old GF or 10-15 weeks old WT C57BL/6J and C3H/HeO_uJ
16 adult male mice). GF mice were obtained from the UCSF Gnotobiotic Facility. In
17 accordance with Biosafety Level 2 guidelines from the Centers for Disease
18 Control and Prevention, GF mice were maintained in sterile gnotobiotic isolators
19 under a strict 12hr light cycle and were fed an autoclaved standard diet.
20 Commercially purchased mice were allowed to acclimatize to their new housing
21 for at least 1 week before any experiments were conducted on them.

22

1 **Human lung tissue samples**

2 Lung tissue samples were collected through an associated lung cancer
3 screening and treatment program, where non-cancerous (“healthy”) portions of
4 the resected lung tissue were excised for this purpose from patients undergoing
5 surgery as part of their definitive lung cancer treatment. The UCSF Institutional
6 Review Board approved the protocols and performance of this study, and written
7 informed consent and Health Insurance Portability and Accountability Act
8 (HIPAA) was obtained from all patients. For metabolomic analyses, lung tissue
9 was immediately frozen on dry ice and stored for later submission for
10 metabolomic analyses (see below). Lung tissue for RNAScope was either fixed
11 right away in formalin or equivalent sections were incubated in PBS or LPS for 2h
12 prior to fixation (see RNAScope section below for more details for subsequent
13 steps).

14 Human rejected donor lungs were obtained with explicit approval for the use
15 of donor lungs for research from each donor’s family by Donor Network West. All
16 donor lung tissue originated from the right upper lobe of the rejected lungs.

17

18 **Reagents and cell lines**

19 Short-chain fatty acids (SCFAs) (acetate, butyrate, propionate),
20 Lipopolysaccharide (LPS), were purchased from Sigma-Aldrich, St. Louis, MO,
21 United States. Seahorse XF calibrant solution (part number 100840-000),
22 Seahorse XF RPMI medium, pH 7.4, (part number 103576-100), and Seahorse

1 XF cell mito stress test kit (part number 103015-100, contains 1 each of
2 oligomycin, FCCP, and rotenone/antimycin A. Oligomycin, FCCP, antimycin A
3 and rotenone) all purchased from Agilent Technologies, Inc.

4 The following cell lines were used in this study: MLE 12 (mouse lung
5 epithelial AT2), MH-S (wild-type BALB/c alveolar macrophage cell line, ATCC),
6 EOMA (129 background endothelial cell line, ATCC, Manassas, VA, United
7 States), HUVEC (primary human umbilical vein endothelial cells, used at
8 passage 6 or less, Promocell, Heidelberg, Germany), HPMEC-M or HPMEC-F
9 (primary human pulmonary microvascular endothelial cells-male or female, used
10 at passage 2–6, Promocell, Heidelberg, Germany). Human lung alveolar
11 macrophages (AM) were collected by bronchoalveolar lavage (BAL) and alveolar
12 type II (AT2) cells were isolated from elastase-digested human lung tissue using
13 negative selection (anti-CD14 and IgG antibodies) (15). All cells were incubated
14 at 37°C under humidified 5% CO₂ and then treated with 10ng/mL or 100ng/mL
15 LPS for 24h or fresh cells were used.

16

Bacteroides thetaiotaomicron (*B. theta*) strains inoculation experiment

18 Two *B. theta* strains: *B.theta* Delta TDK (WT) and *B.theta* Delta 1686-89 (del
19 propionate) strains were a kind gift from Eric Martens, University of Michigan (24,
20 30). Both *B. theta* strains were grown in brain-heart infusion (BHI) broth in an
21 anerobic chamber. Both *B. theta* strains were used to gavage the GF adult
22 (gnotobiotic) C57BL/6 mice and each individual mouse was colonized using

1 100ul of a bacterial suspension with each mouse receiving effectively $\sim 10^9$ CFUs.
 2 Two weeks later of inoculation, samples of lung tissue, fecal pellets and cecal
 3 contents were collected and were homogenized and serially diluted used for
 4 endotoxin content measurement.

5

6 **Endotoxin measurement**

7 Limulus Amebocyte Lysate (LAL) test cartridges (Endosafe® nexgen-PTS™
 8 Charles River Laboratories) – a rapid, point-of-use handheld spectrophotometer
 9 that is used for real-time endotoxin testing – was used in this study for mice lung
 10 tissue and stool/intestinal tissue sample endotoxin measurement.

11

12 **SCFA and Amino Acid Measurement**

13 Short chain fatty acids of human lung samples were quantified using a
 14 Water Acquity uPLC System with a Photodiode Array Detector and an
 15 autosampler (192 sample capacity) at the Baylor University Metabolomic Core.
 16 Samples were analyzed on HSS T3 1.8 mm 2.1 150 mm column. Flow rate: 0.25
 17 mL/min, injection volume: 5 mL, run-time: 25 min per sample. Eluent A was 100
 18 mM sodium phosphate monobasic, pH 2.5, eluent B was methanol, the weak
 19 needle wash was 0.1% formic acid in water, the strong needle wash was 0.1%
 20 formic acid in acetonitrile, and the seal wash was 10% acetonitrile in water. The
 21 gradient was 100% eluent A for 5 min, gradient to 70% eluent B from 5 to 22 min,
 22 and then 100% eluent A for 3 min. The photodiode array was set to read

absorbance at 215 nm with 4.8 nm resolution. Samples were quantified against standard curves of at least five points run in triplicate. Standard curves were run at the beginning and end of each metabolomics run. Quality control checks (blanks and standards) were run every eight samples. Results were rejected if the standards deviate by greater than 5%. Concentrations in the samples were calculated as the measured concentration minus the concentration of the solvent; the range of detection was at least 1 – 100 mmol/g stool. Stool samples were similarly analyzed for SCFA levels. Some samples were also sent independently to University of Michigan Metabolomic core to confirm findings and representative data is shown.

Seahorse™ Extracellular Flux Metabolism Analysis

To examine metabolic response in the MH-S alveolar macrophage (AM) cell lines exposed to LPS and SCFAs, Agilent Seahorse XFe24 analyzer platform (Agilent Technologies, Inc.) was used per manufacturer's instructions. Briefly, MH-S cells were seeded into XFe24 cell culture plate, 40K/well were grown in RPMI medium supplemented with 10% FBS and 1% P/S. All treatments given 4h after seeding cells. As noted, cells were treated with combinations of LPS (10ng/mL), acetate (5mM), propionate (0.1mM or 5mM).

RNA Scope™ *In Situ* Hybridization for RNA transcript detection

Lungs from naïve wild-type C3H mice or mice that underwent LPS

1 challenge (10mg/kg IV for 1 h) (41, 51) were fixed in formalin. Human lung tissue
2 was obtained following a lobectomy for tumor resection; the section studied was
3 the furthest section away from the isolated nodule and thus reflective of non-
4 tumor/"normal" lung. Lung sections were either fixed in formalin immediately
5 (fresh) or incubated in PBS or 10µg/mL LPS solution for 2h before fixing. Paraffin
6 embedded sections were stained using RNAScope® (from Advanced Cell
7 Diagnostics – ACDBio) using specific human or mouse RNA probes for IL-1β, IL-
8 18, and for cell markers, such as hopx (AT1 cells), surfactant C (AT2 cells),
9 CD11c (AM and dendritic cells), CD45 (hematopoietic cells), CD206/MRC1
10 (macrophages). Probes were visualized as two colorimetric dyes (dots or
11 clusters) via light microscopy (mouse) or three fluorescent dyes via fluorescent
12 microscopy (human). Briefly, 5 µm formalin fixed, paraffin- embedded tissue
13 sections were pretreated with heat and protease prior to hybridization with the
14 target oligo probes. Preamplifier, amplifier and AP-labeled oligos were then
15 hybridized sequentially, followed by chromogenic precipitate development. Each
16 sample was quality controlled for RNA integrity with a positive control probe
17 specific to the housekeeping gene peptidylprolyl isomerase B and for background
18 with a negative control probe specific to bacterial *Bacillus subtilis* gene
19 dihydrodipicolinate reductase (dapB). Optimization of pretreatment conditions
20 was performed to establish the maximum signal to noise ration. Specific RNA
21 staining signal was identified as black, red, or blue-green punctate dots. Samples
22 were counterstained with Gill's Hematoxylin with nuclei appearing light purple or

1 DAPI.

2

3 **Quantitative reverse transcription real-time polymerase chain reaction**

4 TaqMan-specific inventoried gene primers for glyceraldehyde 3-phosphate
5 dehydrogenase (GAPDH), beta actin, β -glucuronidase (GUSB), interleukin (IL)-
6 6, IL-1 β , chemokine (C-X-C motif) ligand (CXCL) 1, CXCL2, free fatty acid
7 receptor (FFAR) 2, FFAR3, and toll like receptor (TLR) 4 were used to measure
8 the message levels of these human or mouse genes in cells or lung tissue (Life
9 Technologies, Carlsbad, CA).

10 Lung tissue was homogenized (Tissue-Tearor - Biospec Products,
11 Bartlesville, OK) and total RNA isolated using Trizol[®]. We used the High Capacity
12 RNA-to-cDNA reverse transcription Kit using 1 μ g messenger RNA per reaction
13 (Life Technologies, Carlsbad, CA). Quantitative real-time polymerase chain
14 reaction was performed using the ABI Prism 7000 Sequence Detection System
15 (Life Technologies, Carlsbad, CA). Run method: Polymerase chain reaction
16 activation at 95°C for 20s was followed by 40 cycles of 1s at 95°C and 20s at
17 60°C.

18 The average threshold counts (Ct) value of 2–3 technical replicates were
19 used in all calculations. The average Ct values of the internal controls
20 (glyceraldehyde 3-phosphate dehydrogenase, beta actin) was used to calculate
21 Δ Ct values for the array samples. Data analysis was performed using the $2^{-\Delta \Delta Ct}$
22 method, and the data were corrected for statistical analysis using log

1 transformation, mean centering, and autoscaling.

2

3 **Sandwich enzyme-linked immunosorbant assay (ELISA)**

4 Concentrations of IL-6 in cell culture supernatant were determined using the
5 mouse DuoSet kit (R&D Systems, Minneapolis, MN). All assays were performed
6 according the manufacturer's supplied protocol. Standard curves were generated
7 and used to determine the concentrations of individual cytokines or chemokines
8 in the sample. Results are presented as Mean $\pm$ SD.

9

10 **Statistical Analysis**

11 Data in the figures are expressed as mean $\pm$ SD. Data from *in vivo*
12 studies comparing two conditions were analyzed using 2-tailed nonparametric
13 Mann–Whitney analyses. Data from *in vitro* studies comparing two conditions (*ex*
14 *vivo* pooled alveolar macrophage stimulation studies) were analyzed using
15 standard Student's t-test with equal SD to generate *P* values. For analyses of
16 multiple groups (such as for the time course experiments in Figure 2), simple 1-
17 way ANOVA was used and Tukey's correction for multiple comparisons applied.
18 GraphPad Prism was used for statistical analyses (GraphPad Software, La Jolla,
19 CA). For all *in vivo* experiments, exact *P* values are reported, and for *in vitro*
20 analyses, *p* values < 0.05 were considered significant. *P* values are represented
21 as follows in the figures: * < 0.05 ; ** < 0.01 ; *** < 0.001 ; **** < 0.0001 . Experiments
22 were repeated 2 or more times, as indicated in the figure legends. If any samples

- 1 were excluded from analysis, the number of samples excluded and the reasons
- 2 for exclusion are included in the figure legends for the corresponding figures.

3

4

1 RESULTS

2 *Human lung tissue contains variable micromolar levels of acetate and propionate.*

3 Human lung tissue from 5 patients undergoing lung lobectomies were assayed
4 SCFA (including medium and branch-chain fatty acids: C2-C8) levels. We noted
5 significant variability in the relative levels of all SCFAs between patients (**Figure**
6 **1A**) as well as when comparing the absolute levels of the SCFAs (**Figure 1B**,
7 left) or as a percentage of total SCFAs (**Figure 1C**, left). Previously, we had
8 reported that the ratios of C2:C3 can regulate lung inflammatory responses (50)
9 and these varied from 3:1 to 10:1 in this cohort (**Figure 1B**, right and **Figure 1C**,
10 right).

11

12 *LPS and SCFA presence in naïve murine lungs depends on the presence of an*
13 *intact gut microbiome.*

14 To confirm that the gut microbiome was the source of lung SCFAs, we examined
15 the metabolomic profile of colons/stool and lung tissue of mice that originated
16 from UCSF's germ-free (GF aka gnotobiotic) and SPF (specific pathogen free,
17 conventionally housed) facilities. We observed that GF mice had low to absent
18 levels of SCFAs (C2-8) confirming wide-spread literature reports that SCFAs are
19 not generated in mammalian cells and are solely derived from metabolically-
20 active and/or fiber-fermenting gut bacterial species (**Figure 2A**). We measured
21 the levels of LPS present in GF versus SPF mouse stool and lung tissue and
22 noted the presence of LPS in the lungs of SPF mice compared to GF mice

(**Figure 2B, right**). As a proof of principle, we mono-colonized GF mice with a gram-negative bacterial species (*B.theta*) and found that this colonization led to an increase in LPS within the intestine compared to GF mice; as well as a large increase in LPS levels (albeit extremely low absolute LPS concentrations) in the lungs of the colonized mice (**Figure 2B**). This is consistent with the amounts of LPS expected to transit from the gut microbiome to the lung as part of the gut-lung axis to establish along with associated SCFA levels, the resting lung immune tone. Finally, we used a *B.theta* mutant (del propionate) that was unable to produce C3/propionate to demonstrate that propionate from the intestine could travel to lung tissue: colonization by gavage of GF mice with C3 mutant *B.theta* resulted in less propionate present in the lungs of mice colonized compared to colonization with wildtype *B.theta* (**Figure 2C**).

SCFAs can alter mouse alveolar macrophage metabolism and responses to LPS exposure.

We had previously reported that SCFA could significantly modulate AM response to LPS (or lipoteichoic acid) (50). Here, we wanted to understand the effects of SCFA and LPS co-presence on lung AM metabolism using Seahorse™ extracellular flux analyzer. We examined the metabolic response (glycolysis and oxidative phosphorylation) of AM to LPS and the effects of co-exposure to SCFA (**Figure 3A, top**). We observed that while low dose LPS exposure reduced maximal respiration and spare respiratory capacity of AM (blue bars), high dose

propionate/C3 (purple bars) could partially reverse these LPS effects (**Figure 3A, bottom left and right**). Next, AMs were subjected to metabolic stress tests and monitored using Seahorse™. We found that AMs exposed to C3 displayed aerobic and energetic metabolic profiles, while LPS challenged AM were more glycolytic (**Figure 3B**). High dose C3, as we noted earlier, was able to partially reverse the metabolic phenotype of LPS-exposed AM (**Figure 3B**). In **Figure 3C** we present the metabolic profiles of AM co-exposed to no LPS and high dose C3 (↑C3) versus those exposed to LPS and low dose C3 (↓C3). As we have previously reported, the former conditions mimic the predicted antibiotic-treated lung environment which resulted in reduced lung inflammation following sterile injury and the significant enrichment of gram-positive propionate-producing *Lachnospiraceae* (51), while the latter conditions mimic the control group with an unperturbed gut microbiome and in whom robust lung inflammation ensues after sterile ischemia-reperfusion (IR) lung injury. Conditions that seemed to favor more inflammation correlated with AM metabolic profiles with lesser metabolic reserve, reduced ability to increase oxygen consumption rate when stressed, and greater extra cellular acidification rate (**Figure 3C, right panels**).

Specific subsets of naïve and LPS stimulated mouse AM subsets express IL-1β.

In order to further understand the effect of the gut microbiome on lung immune tone, we examined the baseline and LPS-stimulated expression of IL-1β within the mouse lung. Using spatial transcriptomic RNA probe pools (RNAScope™) to

detect IL-1 β and surfactant C mRNA, we had previously showed that resting, LPS-induced, and lung IR-induced IL-1 β mRNA was expressed along the alveolar surface and in close proximity to AT2 cells and that IL-1 β was not expressed by AT1 (HopX expressing) cells both in naïve and LPS-challenged lungs (51). Some CD11c⁺ AMs residing within the alveolar space appeared to express the naïve lung IL-1 β signal indicating that a subset of AM may be responsible for IL-1 β -dependent lung immune tone – both in the naïve and LPS-induced lungs (**Figure 4A**). These IL-1 β expressing cells were confirmed to be CD45⁺ (hematopoietically-derived) (**Figure 4B**). Further imaging here revealed punctate IL-1 β mRNA expression in the alveolar space appeared in close conjunction to AT2 cells (Surfactant C expressing) cells (**Figure 4c**) suggesting some type of coordination between AT2 and AMs with regard to IL-1 β lung immune tone/priming. We also probed these lungs for IL-18, a member of the IL-1 β family of cytokines also regulated by the inflammasome. IL-18 has been shown to be important for various lung disease pathologies (1, 26, 36, 58, 59), and could also serve as a potential contributor to lung immune tone. Interestingly, we found that IL-18 was expressed in a separate subset of cells distinct from IL-1 β expressing cell (**Figure 4D**) suggesting perhaps of the existence of IL-1 β and IL-18 specialized AM subsets.

FFAR2 and FFAR3 are expressed in AT2 and AM mouse cell lines and are inducible by LPS.

1 How lungs sense signals transmitted from the gut is not well understood. We
 2 focused on mouse lung cell lines, such as AT2 (MLE-12 cells), AM (MH-S), and
 3 EC (EOMA) and first examined the LPS responsiveness of these cell lines *in vitro*
 4 and found that the EC more than AM were the major sources of IL-6 production
 5 (**Figure 5A**). Baseline expression of IL-1 β and FFAR2 were highest in AM, and
 6 FFAR3 was similarly expressed in AT2 and AM (**Figure 5B**). Low dose LPS (10
 7 ng/mL) resulted in a large increase in AM's IL-1 β , and AT2's FFAR2 and FFAR3
 8 expression (**Figure 5C**). Higher dose LPS (100 ng/mL) further increased the
 9 AM's IL-1 β and FFAR2 expression, and AT2's FFAR3 expression (**Figure 5C**).
 10 Therefore, low level (such as may be seen in naïve mice) and high-level LPS
 11 (such as which could be seen in an infection or dysbiosis) resulted in very
 12 different profiles of FFAR2 and FFAR3 expression in alveolar resident cell types
 13 *in vitro*.

14

15 *Human alveolar cell expression of IL-1 β and FFAR2 and FFAR3 ex vivo vs. in*
 16 *vitro culture.*

17 We measured *in vitro/ex vivo* expression of IL-1 β and FFAR2 and FFAR3 in
 18 human BAL cells (AM) obtained from rejected human donor lungs and found that
 19 cells in culture overnight expressed more IL-1 β and FFAR2 and less FFAR3
 20 mRNA; and LPS exposure *in vitro* led to large and significant increases in all
 21 three gene's expression levels (**Figure 6A**). Comparing freshly isolated AT2 cells,
 22 BAL AM, ECs from male and female cadaveric lungs, and HUVEC, we noted that

1 AT2 and AMs were the primary source of IL-1 β , FFAR2, and FFAR3 mRNA and
 2 not EC *in vitro* (**Figure 6B**). Interestingly, freshly isolated AT2 (versus the same
 3 cells after overnight culture *in vitro*) expressed more IL-1 β message, less IL-6
 4 and consistently low TLR4; in contrast cultured AM expressed more IL-1 β , IL-6
 5 and TLR4 versus freshly isolated BAL AM (**Figure 6C**).

6

7 *Human cellular expression of IL-1 β and FFAR2 and FFAR3 in situ in fresh*
 8 *human lung tissue and BAL AMs.*

9 Our previous results suggested that alveolar cells outside of their natural lung
 10 microenvironment context may have very different expression of IL-1 β , FFAR2,
 11 and FFAR3 and perhaps other genes. Therefore, we examined expression of
 12 these genes along with cell-specific markers *in situ* in the human lung. We noted
 13 that IL-1 β signal in naïve human lungs was present in a subset of AMs that were
 14 CD11c/ITGAX positive (**Figure 7A**) and CD206/MRC1 positive (**Figure 7B**).
 15 Once again, we noted the close proximity of naïve lung IL-1 β mRNA signal to
 16 AT2 cells (SFTPC/surfactant C positive (**Figure 7A**, far right inset b). FFAR2 and
 17 FFAR3 were co-expressed in MRC1/CD206 positive AMs as well as in other yet
 18 unidentified cell types (**Figure 7C**) but not by AT2 cells (**Figure 7D**). We exposed
 19 sections of the human lung tissue to LPS for 2h to stimulate the expression of IL-
 20 1 β and found that a subset of AMs which were ITGAX/CD11c positive and
 21 CD206/MRC1 positive expressed IL-1 β both at baseline and after LPS challenge
 22 (**Figure 7E-F**). To assess the level of variability in human lung immune tone, we

1 assayed two different donor lung BAL cells for IL-1 β , FFAR2 and FFAR3
2 expression and found 2-4 orders of magnitude differences in all three genes
3 mRNA (**Figure 7G**). Moreover, the relative levels of these three genes seemed to
4 change similarly implying a possible coordinated expression of this gene set.

5

6 **DISCUSSION**

7 The major conclusions of this study are that human lungs contain
8 metabolites and other bacterial signals that originate from the intestine, and that
9 these signals likely contribute to the immune tone of the lung, namely the resting
10 readiness state that the lungs exist in prior to injury or infection. These
11 conclusions are based on the following experimental evidence. First, we
12 demonstrated SCFA metabolites exist in human lung tissue and confirmed in
13 mice that lung SCFA levels depended on intact and functional gut microbiota.
14 Next, we demonstrated that the metabolic effects of LPS exposure on lung
15 macrophages was profoundly affected by the co-presence of SCFAs. We then
16 examined naïve and injured mouse lungs and found clear presence of immune
17 tone via IL-1 β expression within alveolar cells, as well as expression of SCFA
18 receptors, namely FFAR2 and FFAR3, in alveolar macrophages (AM) and
19 alveolar type 2 epithelial (AT2) cells. Exposure to LPS also regulated the
20 expression of FFAR2 and FFAR3. Confirming these findings in human lung cells,
21 we detected IL-1 β , FFAR2 and FFAR3 expression in alveolar cells *in vitro* and *in*
22 *vivo*. Collectively, this experimental evidence strongly supports the concept of

1 lung immune tone as defined by the basal expression of pro-IL-1 β message
2 (Inflammasome signal 1), which is regulated by the gut microbiome through
3 transport of bacterial products, including metabolites and LPS, along the gut-lung
4 immune axis.

5 We previously demonstrated through the use of antibiotics in mice that the
6 gut microbiome regulates sterile lung inflammation (42). Furthermore, we
7 reported that IL-1 β and its downstream regulation by the NLRP3 inflammasome
8 was required for both sterile inflammatory and infectious immune responses (51).
9 Finally, we uncovered a connection between propionate-producing gut bacteria
10 and reduced sterile lung inflammation and observed that direct propionate
11 introduction into the lung reduced lung anti-bacterial defense (50). Other groups
12 have also described the gut-lung axis [reviewed in (7, 11)]. The Marsland and
13 Lynch groups have published seminal work suggesting strong indirect and direct
14 effects of the gut microbiome on allergic asthma in mice and humans (17, 31, 33,
15 53). Our study focused on the direct role of metabolites on influencing lung
16 inflammation (18, 31). FFAR2 and FFAR3 have recently been reported in
17 multiple cells in lung tissue including airway smooth muscle (35), airway
18 epithelium (23, 54), mesenchymal cells (44), neutrophils, and alveolar
19 macrophages (19). We speculate that immune, metabolic, and other regional
20 heterogeneity within each lung may reflect the need for the lung to
21 compartmentalize its various functions and to allow gas exchange, inflammation
22 generation and resolution, and housekeeping/regeneration to occur

1 simultaneously and this has been supported by recent reports (20, 56). Finally,
2 that SCFAs can contribute to immune tone is supported in the literature by
3 reports that SCFAs have pro-inflammatory effects (27), that LPS up-regulates
4 FFAR2 and FFAR3 (2), and that acetate (via FFAR2) affects type I interferon
5 responses after RSV infection (3).

6 We chose to focus on the gut microbiome and not the lung/airway
7 microbiome based on the high metabolic activity that would be required for SCFA
8 production. The distal ileum and colon contain a million to billion fold more
9 bacteria per gram of organ weight in the distal ileum and colon as compared to
10 the healthy lung (45). Many groups have strongly supported the role of the lung
11 microbiome in influencing ARDS, sepsis and other lung disease outcomes (4, 12,
12 13, 38). This may be due to a greater microbial biomass within context of
13 diseased lungs (where favorable growth conditions may exist) and due to gut
14 bacterial translocation to the lung in critical illness [reviewed in (32, 55)].
15 Additionally, the effects of the gut microbiome and SCFAs on T_{reg} development
16 and dendritic cell function locally in the colon, and remotely in the lung, has been
17 shown to also contribute to the gut-lung axis (48, 53, 57, 60). Our experiments
18 examined baseline steady-state immune tone in healthy lungs and immediate
19 injury responses when gut-originating SCFAs likely have the strongest influence
20 on early lung inflammatory responses. Also, while we focused in this study on
21 SCFAs signaling via FFARs, attention should be also given to the suppressive
22 effects of histone deacetylase (HDAC) inhibition on how propionate and butyrate

1 mediate inflammatory responses (9). Notably, HDAC inhibition requires millimolar
2 concentrations of SCFA usually seen in the colon (vs micromolar lung SCFA
3 levels). Additionally, FFAR2-independent effects of SCFAs have been reported in
4 lung eosinophils and mast cells in asthma and allergic airway disease (16, 49).

5 The concept of lung immune tone may be important to consider in field of
6 lung biology. In the past, it was understood that as people age, their lungs were
7 exposed to numerous potentially injurious factors and with each exposure/injury,
8 the lung may heal and/or assume a “new normal” state/immune tone which would
9 in turn influence the response to subsequent damage. The idea that the gut
10 microbiome produces factors that signal to distant barrier organs such as the
11 lung, to influence its health and host defense capability is very intriguing, as a
12 means to understand varied responses to similar forms of lung injury. In severe
13 systemic disease, the phenomenon of the intestinal barrier disruption or ‘leaky
14 gut’ is well known but the influence of intestinal contents in health is less familiar.
15 In fact, the immune tone of other organs, such as the placenta, has been
16 proposed to be influenced by gut microbiome-derived SCFAs and LPS.
17 Dissecting the rheostat-like dynamic control of lung inflammatory and immune
18 responses could lead to better understanding of this baseline lung immune tone.

19 Our study has limitations: we focused on the two major SCFA receptors,
20 namely FFAR2 and FFAR3, and not on others, such as GPR109a and OLFR78
21 (10). GPR109a primarily resides in the gut where its primary ligand (butyrate) is
22 present (25), and the olfactory chemo sensor OLFR78, which binds propionate,

1 acetate and lactate, is found in the upper airway only (8, 10, 39, 52). Other
 2 metabolites, such as 12,13-dihydroxy-9Z-octadecenoic acid (12, 13-diHOME)
 3 and others including primary and secondary bile acids, may also participate in
 4 the gut-lung immune axis and establishment of lung immune tone (31). Another
 5 limitation is the lack of definitive findings that interrupting the gut-lung immune
 6 axis and lung sensing of SCFAs has clear effect on lung inflammatory and
 7 immune responses. Some of these findings have been reported by others,
 8 specifically using global FFAR knockout mice and cells and studying bacterial
 9 pneumonia (19), RSV infection (3), or influenza infection (54). Further studies will
 10 require the generation of lung-specific FFAR knockout mice and careful inhibitor
 11 studies that are topics of future investigations in our laboratory. We have
 12 previously reported that exogenously short-circuiting this gut-lung immune axis
 13 by providing propionate directly to mouse lungs can strongly influence lung
 14 immunity to bacterial pneumonia (50) and further experiments to understand the
 15 dynamic and mutable nature of lung immune tone will be needed.

16 The major conclusions of this study are that the lung possesses the
 17 capability to sense SCFA metabolites by extension the gut microbiome, can
 18 influence and regulate lung immune tone, namely how the lung responds to injury
 19 and infection. Moreover, this effect on immune tone may be driven by changes in
 20 cellular metabolism as a result of local SCFA concentrations. The implication of
 21 these findings are that diet, medication, and lifestyle influences on the gut
 22 microbiome may indirectly have substantial effects on lung inflammatory and

1 immune responses. Moreover, these effects may be more amenable to beneficial
2 intervention via specific SCFA supplementation, diets including fermentable fiber
3 or fecal microbiota transplantation (FMT). Overall, the gut-lung immune axis, not
4 unlike many other axes connecting the gut microbiome to vital organ systems,
5 may be an avenue towards better understanding lung biology in health and
6 disease as well as integrating known effects of genetics, diet and environment on
7 gut and lung function.

8

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16

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1 Figure Legends:

2

3 **Figure 1. Large variability exists in SCFA levels in human lung tissue from**
 4 **different patients and even within a human lung single lobe.** (A) Relative
 5 levels of SCFAs from human lung tissue (n = 5 patients: Patient 0001, 0002,
 6 0003, 0004, and 0008) obtained from surgical resections (lung lobectomies) for
 7 isolated tumor nodules. (B) Absolute concentrations of SCFAs in each of the lung
 8 sections (left) and focusing only on C2-4 (right). (C) Presented as a percentage
 9 of total SCFAs (all SCFAs, left and only C2-4, right). SCFAs (C2-4) are
 10 highlighted by the red box. SCFA = short chain fatty acid, C2 = acetate, C3 =
 11 propionate, C4 = butyrate, iC4 = isobutyrate, C5 = valerate, iC5 = isovalerate, C6
 12 = hexanoate, iC6 = isopropyl hexanoate, C7 = heptanoate, C8 = octanoate, RUL
 13 = Right Upper Lobe.

14

15 **Figure 2. Mouse lung tissue SCFA and LPS levels depend on the presence**
 16 **of a gut microbiome that is metabolically active.** (A) Profiling of SCFAs from
 17 SPF and germ free (GF) mice (n=2 littermates, and three independent colon/stool
 18 and lung tissue samples). C2-4 are highlighted by the red box. (B) Endotoxin
 19 (LPS) levels measured in mouse stool and lungs from SPF, GF and GF mice
 20 colonized with WT *B.theta* (~10⁹ CFU). (C) GF mice were colonized with *B.theta*:
 21 either WT (+*B.theta*) or a propionate-production deficient mutant (+*B.theta* del
 22 propionate) (~10⁹ CFU) and SCFA levels were profiled (left). C3 is highlighted by

1 the red box. Measured levels of C2-4 are shown (right top) with a focus on
 2 propionate levels (right bottom). Experiments were repeated 2 times.
 3
 4 **Figure 3. Alveolar macrophage immunometabolic response to LPS can be**
 5 **modulated by SCFAs.** (A) AM cell line (MH-S) was unstimulated (control) or
 6 challenged with LPS (10 ng/ml), 0.1 mM propionate ($\downarrow$ C3), 5 mM propionate
 7 ($\uparrow$ C3), LPS plus 5 mM acetate (LPS + $\uparrow$ C2), LPS plus 0.1 mM propionate (LPS +
 8 $\downarrow$ C3), LPS plus 5 mM propionate (LPS + $\uparrow$ C3) after seeding cells 4 hours.
 9 Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)
 10 were measured under four blocks corresponding each effector injection, basal,
 11 ligomycin (OM), FCCP and rotenone+antimycin-a (Rot/AA) by using agilent
 12 Seahorse XFe24 analyzer platform. (B) Metabolic stress tests were run to
 13 measure mitochondrial respiration to compare metabolic phenotypes and profiles.
 14 Three circled groups represent three different metabolic stressed profiles: (1)
 15 control, $\downarrow$ C3, and $\uparrow$ C3; (2) LPS+ $\uparrow$ C3; and (3) LPS, LPS+ $\downarrow$ C3, and LPS+ $\uparrow$ C2. (C)
 16 Two conditions were contrasted: exposure to a) $\uparrow$ C3 alone and b) LPS+ $\downarrow$ C3. All
 17 data was standardized to total protein using bicinchoninic acid assay (BCA).
 18 Experiments were repeated 2 times.

19
 20 **Figure 4. IL-1 β presence in naïve mouse AM subsets and increased**
 21 **expression after LPS.** Naïve wild-type C3H mice were treated with LPS (10
 22 mg/kg IV for 1h). Lungs were harvested, fixed and stained using RNAscope®

1 (from ACD) using specific mouse RNA probes for IL1 β in combination with (A)
 2 CD11c (AM and dendritic cells), (B) CD45 (hematopoietic cells), (C) surfactant C
 3 (SurfC; AT 2 cells), and (D) IL-18. Dashed lines represent approximate
 4 estimations of cellular outlines of positively staining cells.

5

6 **Figure 5. IL-1 β is made by mouse AMs and FFAR2 and FFAR3 are**
 7 **expressed by AT2 and AMs but differentially.** MLE 12 (mouse lung epithelial
 8 AT2), MH-S (wild-type BALB/c alveolar macrophage cell line AM), EOMA (129
 9 background endothelial cell line EC) were treated with 10 ng/mL or 100 ng/mL
 10 LPS for 24 hours. Concentrations of IL-6 in cell culture supernatant were
 11 determined using the mouse DuoSet kit (A). Total RNA was extracted and
 12 expression of IL-1 β , FFAR2, FFAR3, IL-6, CXCL-1, CXCL-2 was determined
 13 using RT-PCR (B, C). Experiments were repeated 3 times.

14

15 **Figure 6. Human AMs express IL-1 β and FFAR2 and FFAR3.** Rejected human
 16 donor lung underwent bronchoalveolar lavage (BAL) to collect alveolar
 17 macrophages (AMs) or alveolar type II (AT2) cells were isolated from elastase-
 18 digested human lung tissue (from a different rejected human donor lung) using
 19 negative selection (anti-CD14 and IgG antibodies). (A) BAL AMs were assayed
 20 without further treatment (fresh) or treated with 0 ng/ml (control), 10 ng/mL or 100
 21 ng/mL LPS for 24h. (B) Isolated AT2 cells (Fresh AT2), BAL AMs (Fresh AM),
 22 and commercially obtained primary human pulmonary microvascular endothelial

1 cells - male or female (Female or Male Lung EC) or primary human umbilical vein
2 endothelial cells (HUVEC) were assayed for expression of IL-1 β , FFAR2 and
3 FFAR3 by RT-qPCR. (C) Human BAL AMs and AT2 – either freshly isolated or
4 after 24h in culture, female and male lung EC, and HUVEC were assayed for IL-
5 1 β (Hu IL-1 β), IL-6 (Hu IL-6), and TLR-4 (Hu TLR4) expression by RT-qPCR.

6

7 **Figure 7. Human IL-1 β , FFAR2 and FFAR3 are expressed in AMs *in vivo*.** (A-

8 C) Human lung sections were either fixed in formalin immediately after excision
9 from patient and then stained/probed with DAPI and combinations of (A)
10 surfactant C (AT2 cells, green), IL-1 β (yellow), and ITGAX/CD11c (AM and
11 dendritic cells, red); or (B) IL-18 (green), IL-1 β (yellow), and CD206/MRC1
12 (macrophages, red); or (C) FFAR2 (green), FFAR3 (yellow), MRC1/CD206
13 (macrophages, red); or (D) SFTPC (Surfactant C, green), FFAR2 (yellow) and
14 FFAR3 (red); (E & F) Human lungs were fixed immediately (FRESH) or
15 incubated with PBS or LPS for 2h prior to fixation and then stained/probed with
16 DAPI and (E) ITGAX/CD11c (red channel), and IL-1 β (yellow channel); or (F)
17 MRC1/CD206 (red channel) and IL-1 β (yellow channel). (G) Human IL-1 β ,
18 FFAR2, and FFAR3 mRNA expression were determined by RT-qPCR in freshly
19 isolated BAL alveolar macrophages (AMs) from two different rejected human
20 donor lungs.

21

Figure 1

Figure 2

B

0

Figure 3

Figure 4

Figure 5

A

B

C

Figure 6

Figure 7

Figure 7 (cont'd)